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13. SUPPLEMENTARY NOTES

14. ABSTRACT
The overall subject of this project is blast-traumatic brain injury (blast-TBI) and the role of the SUR1-regulated $NC_{Ca\text{-}ATP}$ channel in blast-TBI. The specific objectives of this project include: (1) develop a standardized rat model of blast-TBI to study the direct transcranial effects of blast on the brain, independent of indirect transthoracic effects; (2) determine the role of the SUR1-regulated $NC_{Ca\text{-}ATP}$ channel in blast-TBI; (3) in normal human volunteers, determine the safety of the SUR1 blocker, glyburide (glibenclamide), as it might be used as prophylaxis against blast-TBI.

During the 4th year of this project we completed immunohistochemical analysis of the expression of the SUR1 and TRPM4 and neuroinflammatory markers in the rats subjected to blast-TBI (Objective 1b) induced by the Cranium Only Blast Injury Apparatus(COBIA), developed and calibrated at the beginning of the project (Obj. 1a). We completed evaluation of the prophylaxis treatment with SUR1 blocker, glyburide in the neurobehavioral outcome after blast-TBI (Obj. 1d, c). Results of the experiments show that administration of glyburide improves short term and long term neurobehavioral outcomes after Blast-TBI, and, compared to posttreatment, prophylaxis pretreatment with glyburide is more effective in preventing long term cognitive deficits.

15. SUBJECT TERMS
blast-TBI, sulfonylurea receptor

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
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Page

INTRODUCTION: Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.

The overall subject of this research project is blast-traumatic brain injury (blast-TBI) and the role of the SUR1-regulated NCCa-ATP channel in secondary injury following blast-TBI. The specific objectives of this research project may be summarized as follows:

(1) develop a standardized rat model of blast-TBI, to permit study of direct transcranial effects of blast on the brain, independent of indirect transthoracic blast effects;

(2) using this rat model, determine the specific role of the SUR1-regulated NCCa-ATP channel in blast-TBI, including testing whether block of SUR1 using glyburide (glibenclamide) would show a beneficial effect in blast-TBI;

(3) in normal human volunteers, determine the safety of oral glyburide (glibenclamide) as it might be used as prophylaxis against blast-TBI.

NOTE: Rather than being final, this report covers research activity only in the time period between 07/01/2012 and 07/02/2013 which reflects requested modification of the grant to extent project for one more year without additional funds.

BODY: This section of the report shall describe the research accomplishments associated with each task outlined in the approved Statement of Work. Data presentation shall be comprehensive in providing a complete record of the research findings for the period of the report. Provide data explaining the relationship of the most recent findings with that of previously reported findings. Appended publications and/or presentations may be substituted for detailed descriptions of methodology but must be referenced in the body of the report. If applicable, for each task outlined in the Statement of Work, reference appended publications and/or presentations for details of result findings and tables and/or figures. The report shall include negative as well as positive findings. Include problems in accomplishing any of the tasks. Statistical tests of significance shall be applied to all data whenever possible. Figures and graphs referenced in the text may be embedded in the text or appended. Figures and graphs can also be referenced in the text and appended to a publication. Recommended changes or future work to better address the research topic may also be included, although changes to the original Statement of Work must be approved by the Army Contracting Officer Representative. This approval must be obtained prior to initiating any change to the original Statement of Work.

During last year of the project we concentrated our efforts on the following tasks:

1) Detailed anatomical evaluation of the cells de novo expressing SUR1 protein, correlation between SUR1 and neuronal injury through immunohistochemistry of neurodegenerative and apoptotic proteins, including microglia/reactive astrocytosis.
2) Evaluation of the effect of glibenclamide administered post Blast TBI on the short term and long term neurobehavioral outcomes.
3) Evaluation of the prophylaxis effect of glibenclamide administered prior to Blast-TBI on the short term and long term neurobehavioral outcomes.

Summary of animal use:

In the time period between 07/01/2012 and 07/02/2013,
total rats used was 209, including 14 naïve, 42 sham, 17 exclusions, 108 blasted rats

Overall, primary blast death 44, (including 1 anesthesia death).

For experiments with Post Blast-TBI glibenclamide treatment:

BDC 24.5cm
Total animal usage 106 rats
27 sham, 4 exclusions, 1 anesthesia death, 28 Blast death, 46 treatments.

BDC 29.5 cm
Total animal usage 72 rats
15 sham, 11 exclusions, 15 blast death, 31 treatment.

For experiments with prophylaxis glibenclamide Blast-TBI treatment:

BDC 29.5 cm
Total animal usage 31 rats
14 Naive, 15 treatment, 2 exclusions.

Objective 1a: establish the usable working range for the "intensity-response" relationship between blast intensity and outcome in our blast-TBI model

A detailed description and validation of the Cranium Only Blast Injury Apparatus (COBIA) to deliver blast overpressures generated by detonating .22 caliber cartridges of smokeless powder was published in *J. Neurotrauma* (1). Our published data demonstrate our central thesis that exposure of the head alone to severe blast predisposes to significant neurological dysfunction.

Objective 1b: determine the time course for SUR1 and TRPM4 upregulation and downregulation post-blast-TBI.

SUR1 and TRPM4 are the regulatory and pore-forming subunits, respectively, of the SUR1-regulated $NC_{Ca\text{-}ATP}$ channel, which is the target of glibenclamide (to be studied in Objective 1c and 1d). This channel and its subunits are not normally expressed but are transcriptionally upregulated post-injury, as we recently reviewed.[2] It is thus important to determine the time course for upregulation of the channel, because this determines the treatment window during which glibenclamide treatment needs to be started. Similarly, it is important to determine the time course of downregulation, because this determines the length of time that treatment must be continued post-injury.

In the previous reports we presented data showing that newly expressed SUR1 and TRPMr proteins and corresponding upregulation of the RNA can be detected as early as 4 hour after Blast-TBI and expression levels of these proteins are elevated for up to & days.

Summary of the work done:
In the time period between 07/01/2012 and 07/02/2013, we continued harvesting and processing brain tissues from the rats subjected to the Blast-TBI. The proteins that are being analyzed in the brain tissues are summarized in table 1.

Table 1: Antibodies/markers used for histology, Immunohistochemistry and immunology

Proteins and **number** of brain samples analyzed	Assays	Histology
SUR1 (sulfonylurea-receptor 1) **56**	Tunel	H & E
TRPM4 (melastatin transient receptor potential) **35**	In situ Hybridization	Nissl
GFAP (Glial fibrillary acidic protein) **10**	Immunoprecipitation	
NeuN (neuronal marker) **24**	Western blot	
APP (Beta-amyloid precursor protein) **64**	qPCR	
Casp 3 (caspase 3) **28**	Tissue hemoglobin detection	
SP1 (ischemic, hypoxic marker) **7**		
ED1 (microglial marker) **36**		
MPO (Myeloperoxidase) **7**		
Iba1 (ionized calcium-binding adaptor molecule-1) **7**		
TNFα (tumor necrosis factor alpha) **7**		
IgG (immune-globulin G) **21**		
Lam (Laminin) **19**		
5-HT (serotonin) **21**		
P65 5		
TPH (tryptophan hydroxylase) **13**		
Fluoro-Jade (neurodegenerative marker) **7**		
vWF von Willebrand factor (vessel marker) **5**		

The tissues collected from rats subjected to the Blast-TBI with and without glibenclamide treatment are currently continued to be analyzed for the cellular markers and markers of the neuroinflammation. We expect that result of these analyses will allow to associate neuroinflammatory changes in different brain areas with neurobehavioral deficits observed after Blast –TBI. Additionally we expect that these

analyses will show correlation of the beneficial effect of the glibenclamide in Blast-TBI with reduced neuroinflammation.

Objective 1c: determine the effect of glibenclamide treatment on short-term outcome from blast-TBI.

The rationale for study of glibenclamide is reviewed in detail in the original submission and from our extensive studies done in our lab (ref. 2,3,4,5), as well as the data shown in the previous reports (objectives 1a and 1b).

GLP (Good Laboratory Practice) compliance. Prior to beginning of the final work on Objective 1c and Objective 1d, we have written and certified all required SOP's (Standard Operating Procedures) , all experimental equipment was calibrated and certified. All personnel involved in these experiments underwent GLP training. Training included Pharmaceutical Training seminars on GLP. GLP training was provided by Jeiven Pharmaceutical Consulting Inc. (Scotch Plains, NJ). All drug treatments , Blast-TBI procedures, short term and long term outcome were assessment were performed in GLP compliant manner . The investigators were blinded to the treatment codes during the blast procedure and all the behavior and immunohistochemical analyses.

Post-blast-TBI treatment.

Glibenclamide delivery for post-blast-TBI treatment.

Within 2–3 minutes of Blast-TBI induction, mini-osmotic pumps (Alzet 2002, 0.5 μl/h; Durect Corp.) were implanted subcutaneously that delivered either vehicle (saline plus DMSO), or glibenclamide stock solution (Sigma-Aldrich). To immediately start the treatment an intraperitonial injection was followed the pump insertion 1ml/kg body weight. Stock solutions of glibenclamide (Sigma, St Louis, Mo) were prepared in dimethylsulfoxide (DMSO) (5 mg/mL). Solutions for delivery were prepared by adding stock solution to normal saline (NS) and clarifying the solution as needed using a minimum amount of NaOH to a pH approximately 8 to 8.5. Solutions prepared in this way and stored at 37°C for 48 hours retained >80% efficacy as measured in patch clamp experiments on K_{ATP} channels in insulinoma cells. Infusion doses were delivered using a subcutaneously implanted miniosmotic pump (Model 2001; 1.0 μL/h; Durect Corp, Cupertino, Calif), which was always implanted immediately after Blast-TBI. Vehicle (NS plus DMSO) was used in the control animal group.

Effect of glibenclamide on short-term outcome from Blast-TBI was studied in three animal groups :1) Sham control;2)Blast-TBI with vehicle treatment; 3) Blast-TBI with glibenclamide treatment

Sensory- and vestibulomotor tests performed: Accelerating RotoRod, Beam Walk, and Beam Balance., and Spontaneous Rearing
Pre-training for behavior was performed for baseline, and behavior tests were repeated on 24 hrs, 3 days, 7 days and 14 days post blast.

Results .

Fig.1

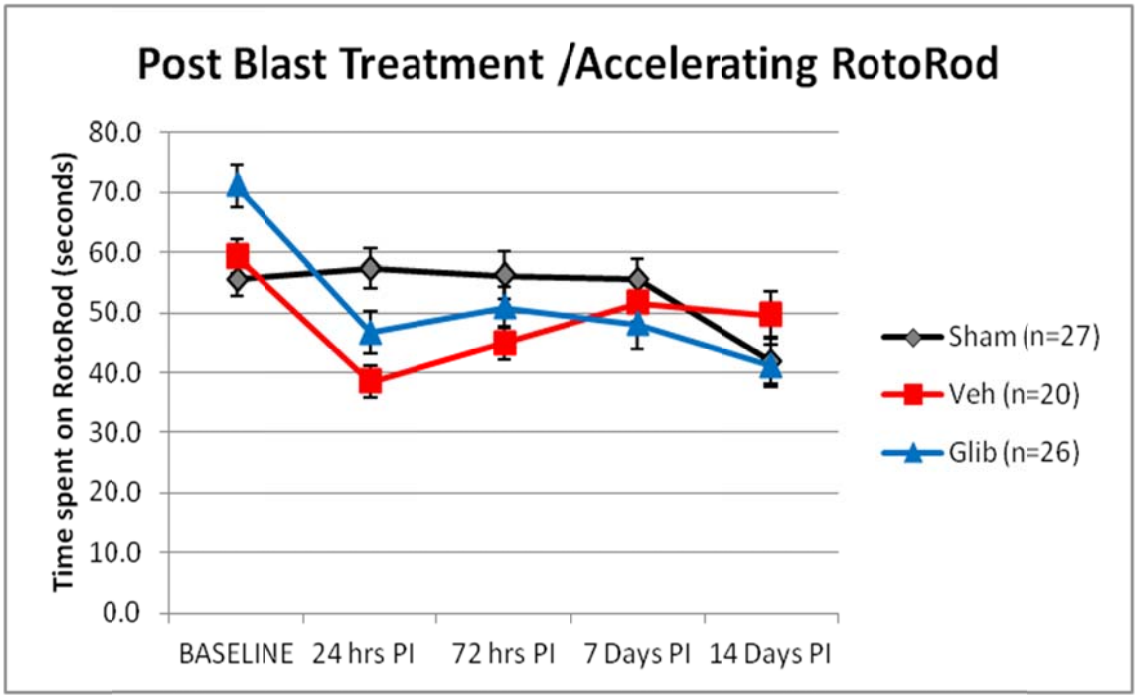


Fig.2

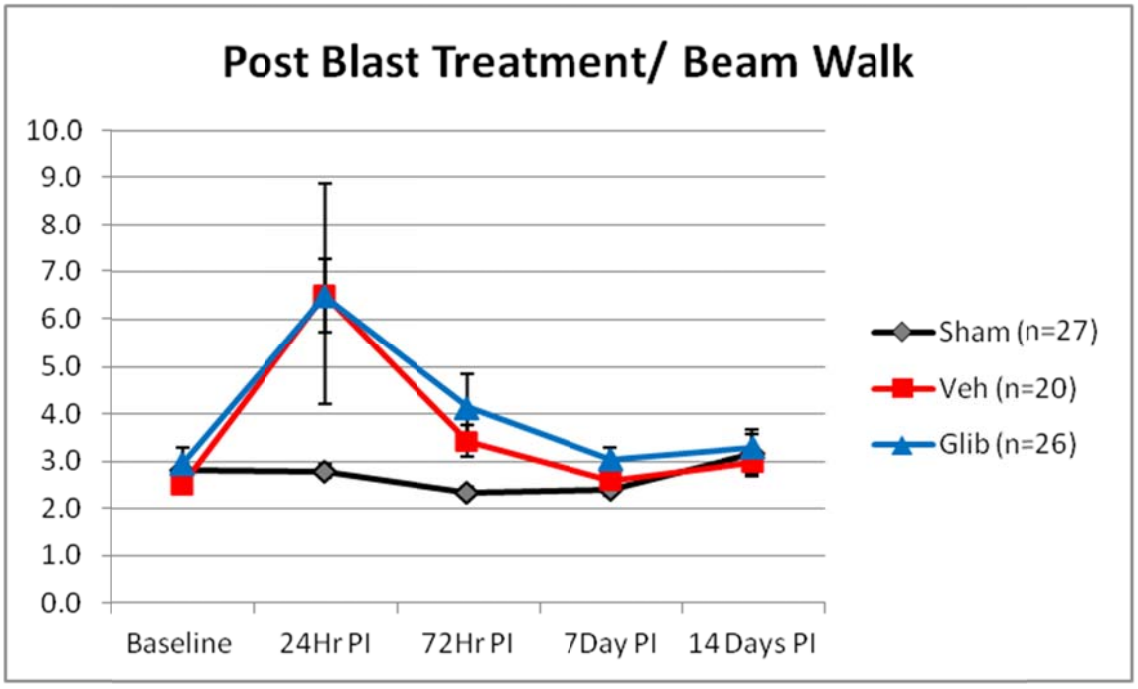


Fig. 3

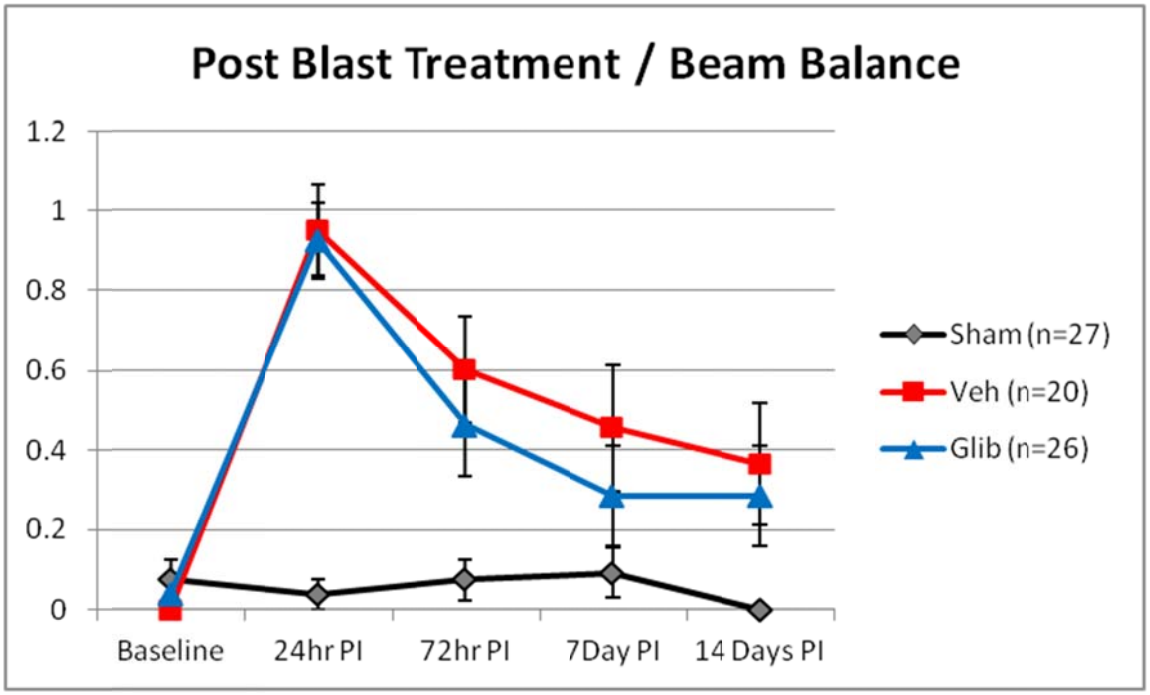
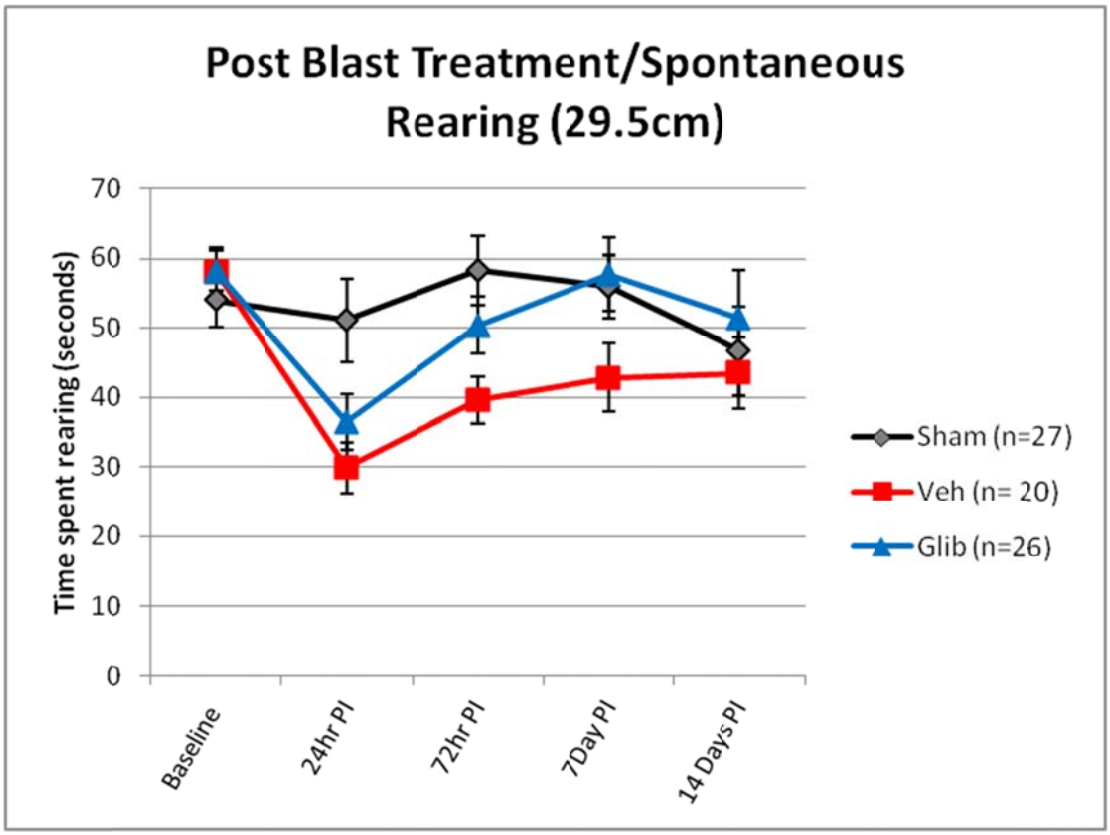

Post Blast Treatment / Beam Balance
1.2
1
0.8
0.6
0.4
0.2
0
Baseline
24hr PI
72hr PI
7Day PI
14 Days PI
Sham (n=27)
Veh (n=20)
Glib (n=26)

Fig.4


Post Blast Treatment/Spontaneous Rearing (29.5cm)
Time spent rearing (seconds)
70
60
50
40
30
20
10
0
Baseline
24hr PI
72hr PI
7Day PI
14 Days PI
Sham (n=27)
Veh (n= 20)
Glib (n=26)

Conclusions.
Cranium only Blast-TBI induced using COBIA apparatus results in transient (1 to 14 days) deficits in non-coerced and coerced vestibulomotor tasks as well as in inhibition of Spontaneous Rearing – vertical exploration behavior , a complex exercise that requires balance, truncal stability bilateral hindlimb dexterity, strength and minimal anxiety. Treatment with glibenclamide administered after Blast-TBI had tendency to improve Beam Balans test outcome (Fig.3) and showed significant faster recovery in Rearing test (Fig.4)

Prophylaxis treatment

Glibenclamide delivery for prophylaxis treatment of the Blast -TBI
Osmotic pumps and composition of the glibenclamide and vehicle solutions were identical to those described above for post-Blast-TBI treatment regimen.
Rats were treated with either vehicle (saline plus DMSO) or glibenclamide one week before the blast and continued treatment for another week after blast. One week before the blast-TBI procedure treatment pumps were implanted subcutaneously, under ketamine/xylazine anesthesia in aseptic conditions . The Blast-TBI was achieved using COBIA with BDC 29.5cm (762kPa) and charge 4 shells. Immediately after the Blast procedure , under anesthesia subcutaneous pumps were exchanged for the new ones providing infusion of the glibenclamide for additional 7 days.
Sensory- and vestibular-motor tests performed: Accelerating RotoRod, Beam Walk, and Beam Balance., and Spontaneous Rearing
Pre-training for behavior was performed for baseline, and behavior tests were repeated on 24 hrs, 3 days, 7 days and 14 days post blast.

Results.

Fig. 5

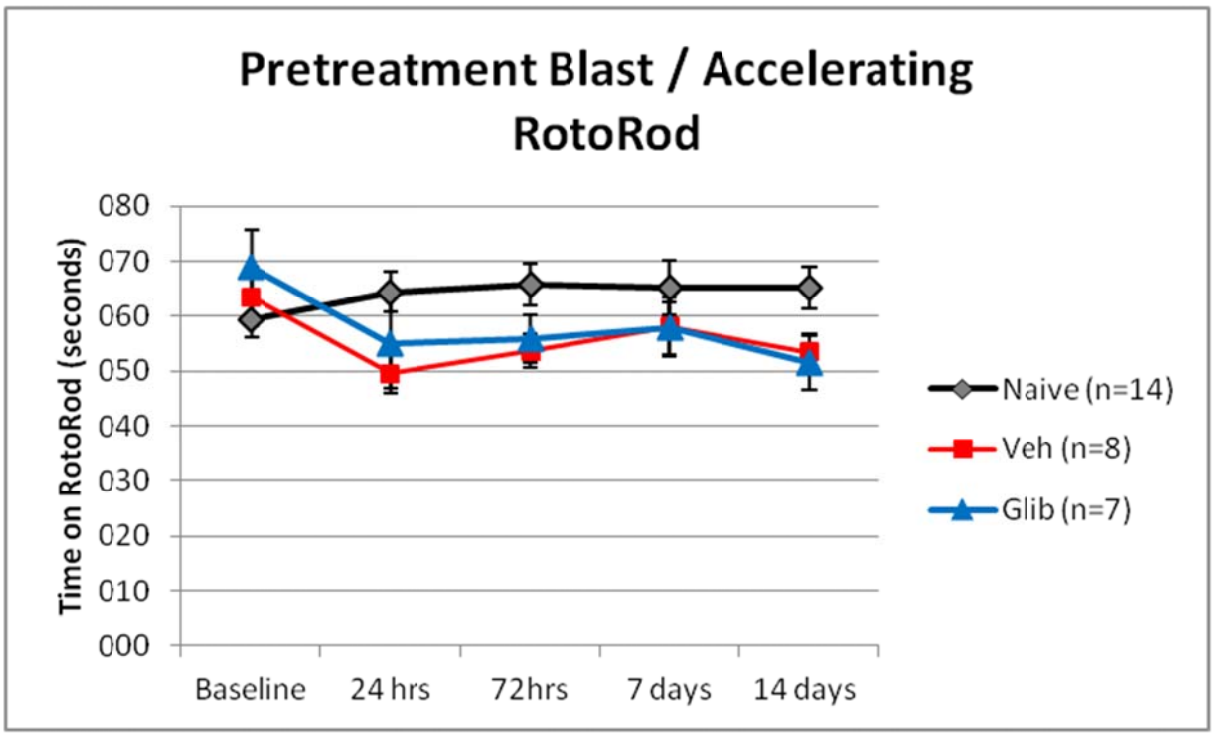


Fig. 6

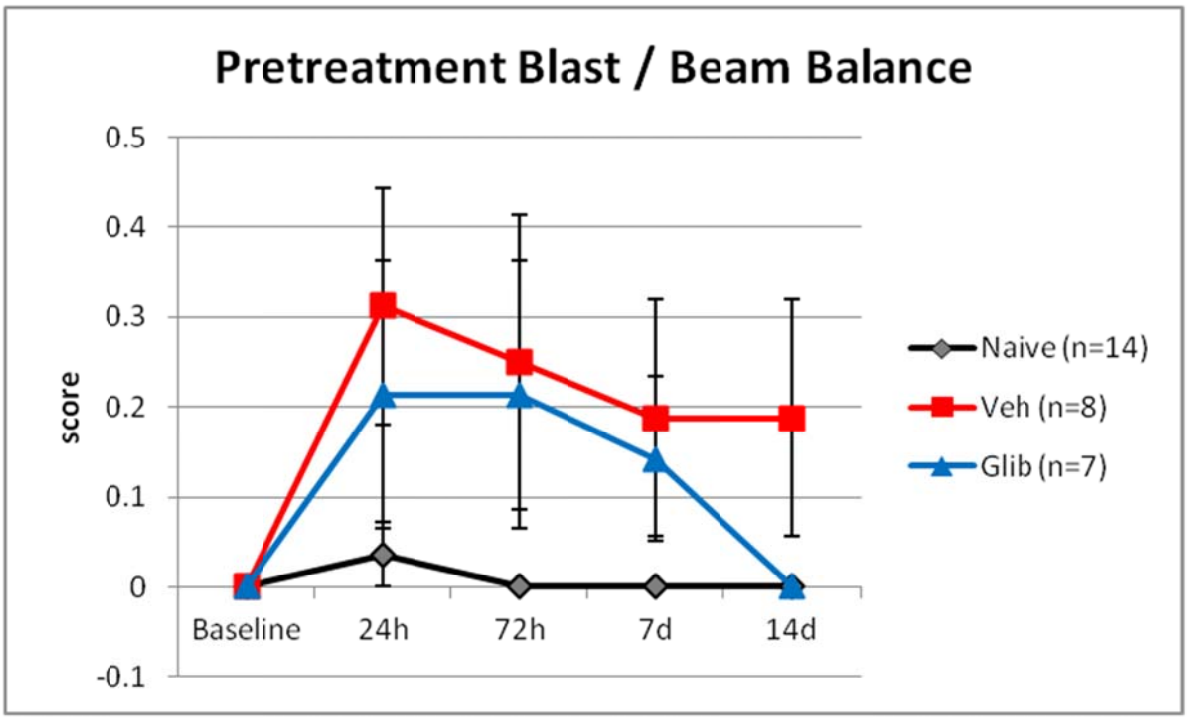
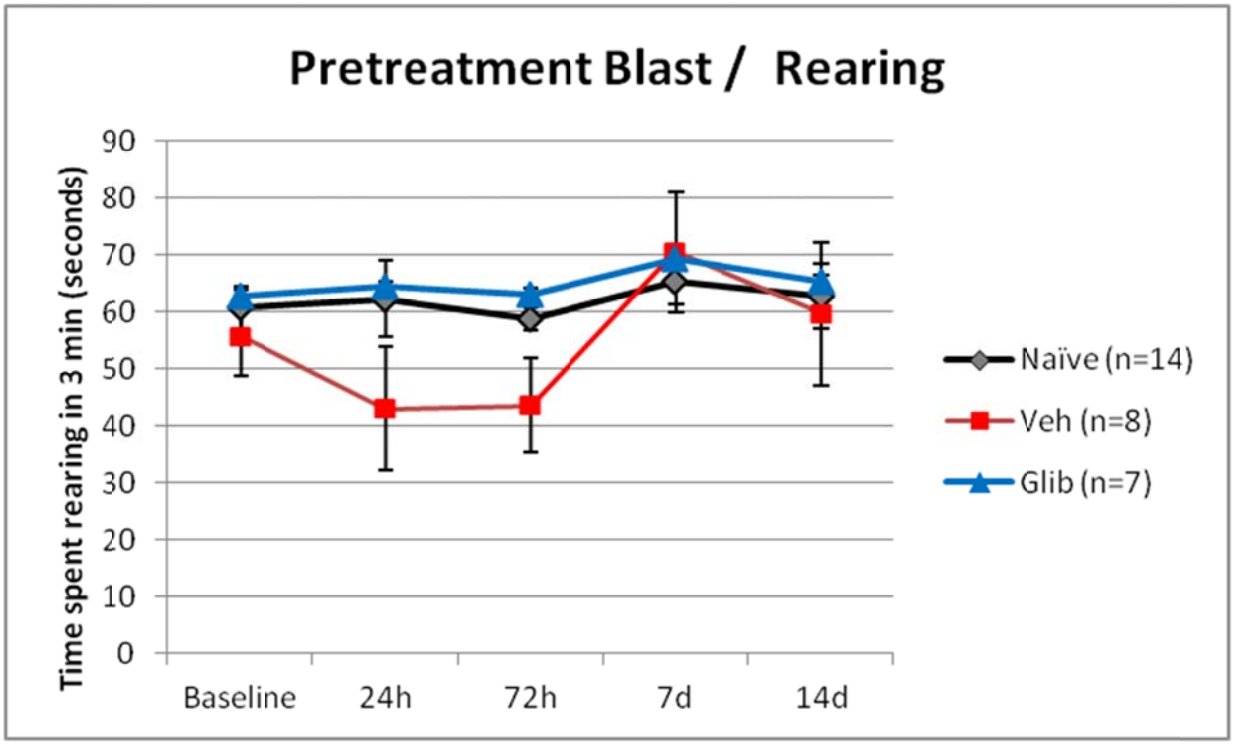

Pretreatment Blast / Beam Balance
score
0.5
0.4
0.3
0.2
0.1
0
-0.1
Baseline
24h
72h
7d
14d
Naive (n=14)
Veh (n=8)
Glib (n=7)

Fig. 7


Pretreatment Blast / Rearing
Time spent rearing in 3 min (seconds)
90
80
70
60
50
40
30
20
10
0
Baseline
24h
72h
7d
14d
Naïve (n=14)
Veh (n=8)
Glib (n=7)

Conclusions. Prophylaxis treatment with glibenclamide prior to the Blast-TBI showed significantly better improvement in vestibulomotor deficits tested in Beam Balance task (Fig. 6) in comparison to post Blast-TBI treatment. In Spontaneous Rearing task vertical exploration activity deficit observed after Blast-TBI with vehicle treatment (Fig. 7, Veh) was absent in rats after Blast- TBI treated with glibenclamide (Fig. 7, Glib)

Objective 1d: determine the effect of glibenclamide treatment on long-term neurobehavioral outcome from blast-TBI

Post-blast-TBI treatment.

Glibenclamide delivery for post-blast-TBI treatment. The glibenclamide treatment regimen is identical to the regimen described above for the study on short-term outcome from blast-TBI (Objective 1c).

Cognitive/learning tests performed: Morris Water Maze (MWM) based: Incremental learning test, Memory Probe test, and Rapid learning Test.
Morris water maze tests were initiated on day 14 after Blast-TBI and were continued for 14 days. After completion, at day 28 all animals were euthanized, perfusion fixed with formalin and brain tissues harvested for immunohistochemical and molecular studies.

Results.

Fig. 8

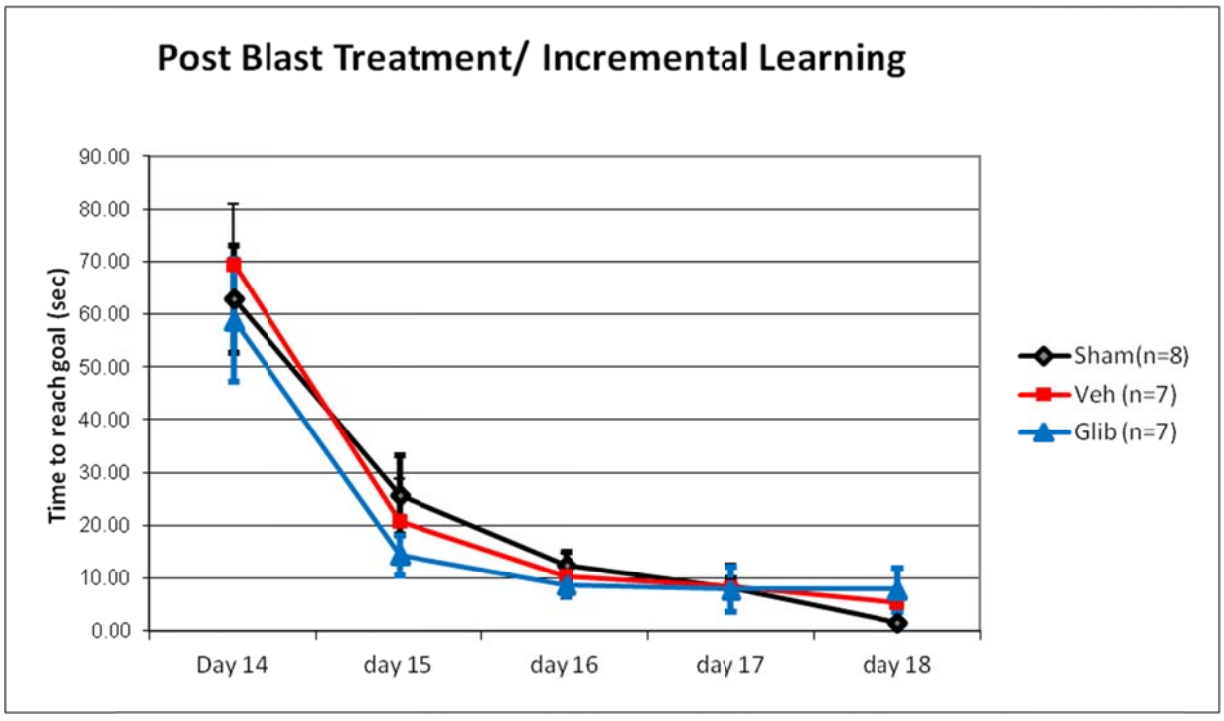


Fig. 9

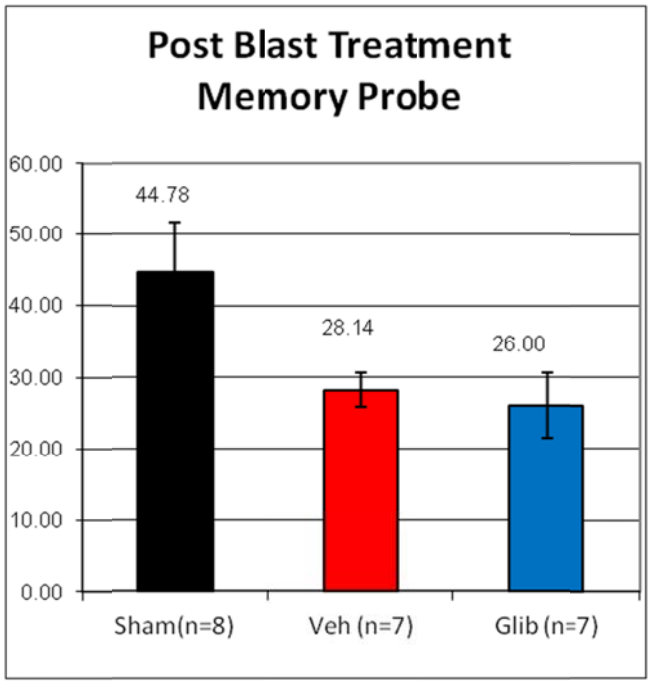
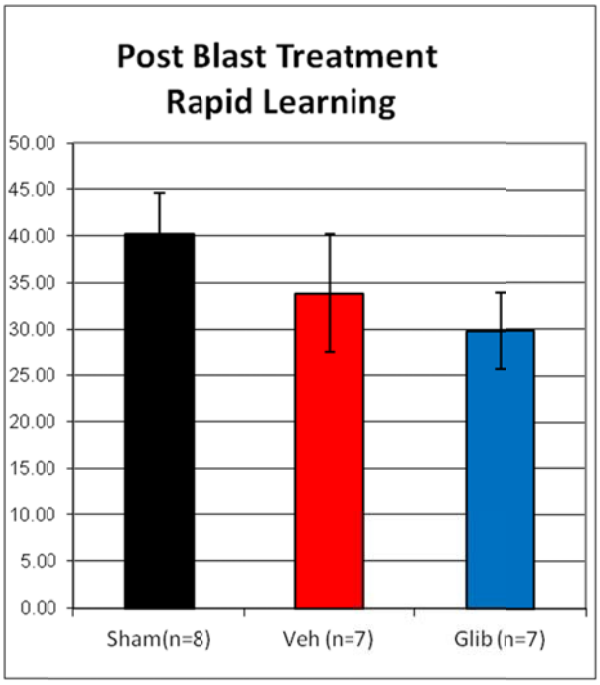


Fig. 10



Conclusions. Analysis of the acquisition and retention of the cued spatial learning using Morris Water Maze tasks show that Blast-TBI did not impede Incremental Learning or acquisition phase (Fig. 8). However, Blast-TBI caused prominent cognitive deficits associated with retention of the learned ability to reach quickly hidden platform (Memory Probe, Fig. 9). Additionally, only sham animals exhibited memory retention for a new hidden platform location after a single learning trial at 4 weeks after injury (Rapid Learning task, Fig. 10). Glibenclamide treatment post- Blast-TBI did not show significant improvements compared to vehicle treated rats in these cognitive/learning tasks.

Prophylaxis treatment

Glibenclamide delivery for prophylaxis treatment of the Blast -TBI The glibenclamide treatment regimen is identical to the regimen described above for the study on short-term outcome from blast-TBI (Objective 1c) except pumps glibenclamide delivery were implanted subcutaneously 1 week prior to Blast-TBI procedure and replaced immediately after to continue glibenclamide infusion for 1 week after .

Cognitive learning tests performed: Morris Water Maze (MWM) based:
Incremental learning test, Memory Probe test, and Rapid learning Test.
Morris water maze tests were initiated on day 14 after Blast-TBI and were continued for 14 days. After completion, at day 28 all animals were euthanized, perfusion fixed with formalin and brain tissues was harvested for immunohistochemical and molecular studies.

Results .

Fig. 11

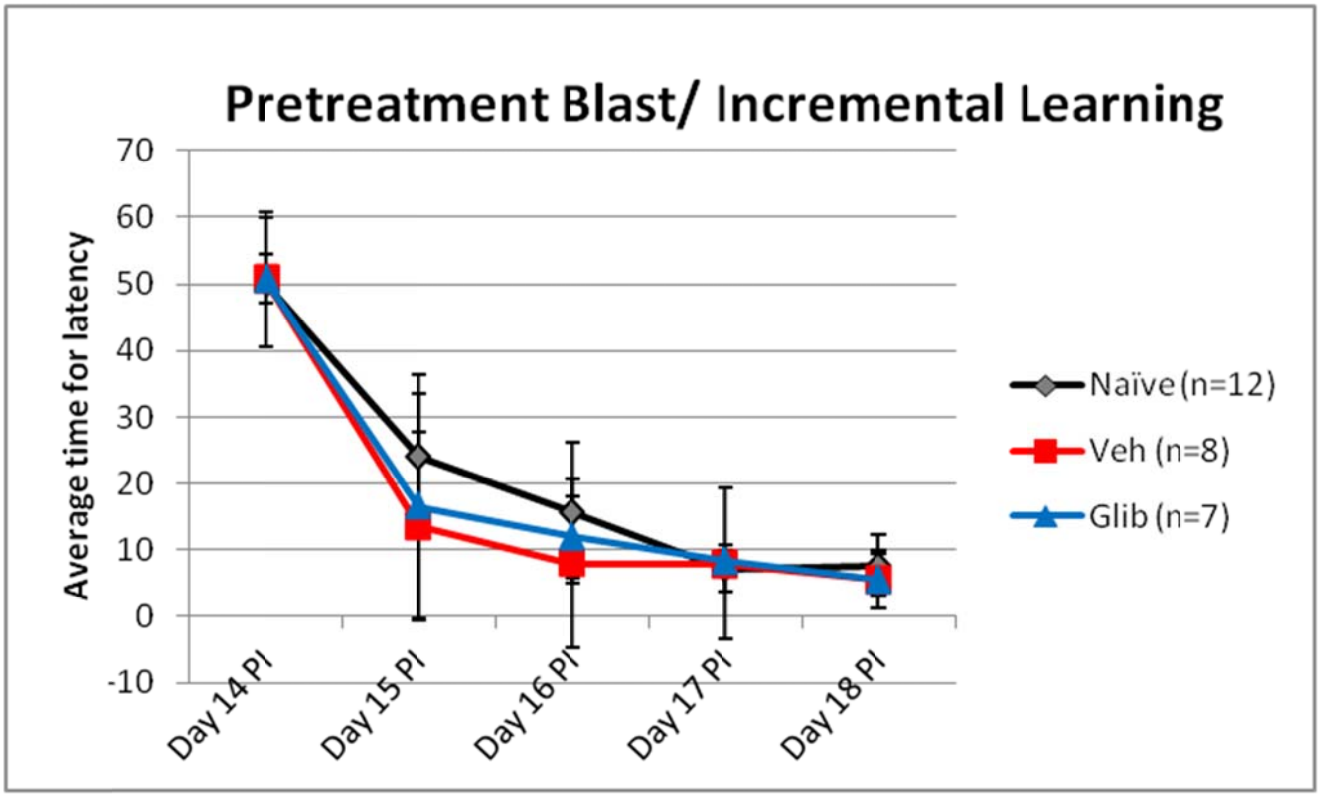


Fig. 12

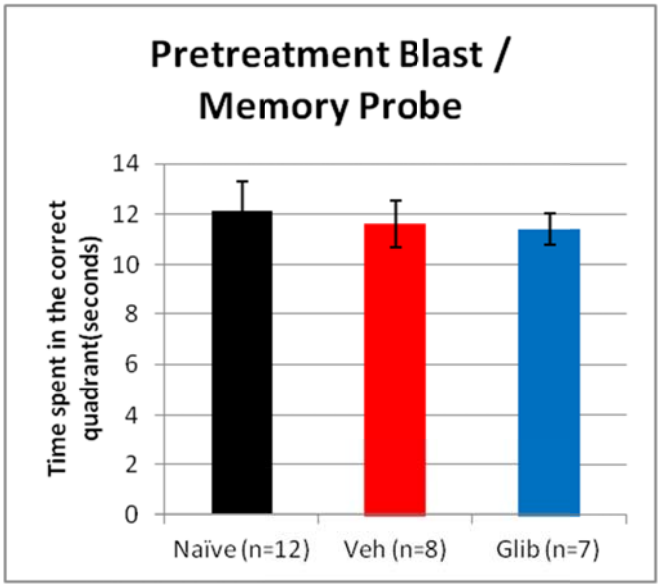
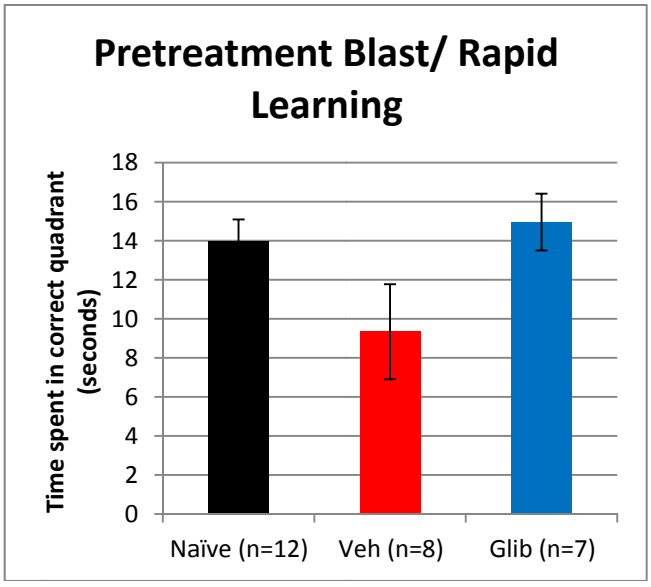


Fig. 13



Conclusions. Analysis of the acquisition and retention of the cued spatial learning using Morris Water Maze tasks show that Blast-TBI did not impede Incremental Learning or acquisition phase (Fig. 11). However, Blast-TBI caused prominent cognitive deficits associated with retention of the learned ability to reach quickly hidden platform (Memory Probe, Fig. 9). Notably, both sham and Blast-TBI glibenclamide pretreated but not vehicle treated animals exhibited memory retention for a new hidden platform location after a single learning trial at 4 weeks after injury (Rapid Learning task, Fig. 13). Glibenclamide prophylaxis treatment of the Blast-TBI did show significant improvements compared to vehicle treated rats in these cognitive/learning tasks (Fig. 13).

OBJECTIVE 3: in normal human volunteers, determine the safety of oral glibenclamide as it might be used as prophylaxis against blast-TBI.

The SUR1 blocker, glyburide, in normal human volunteers. This activity has been transferred to the University of Washington, St. Louis, due to the fact that the investigator responsible for this experiment has moved there as faculty.

KEY RESEARCH ACCOMPLISHMENTS: Bulleted list of key research accomplishments emanating from this research.

During last year of the project we concentrated our efforts on the following tasks:

1) Detailed anatomical evaluation of the cells de novo expressing SUR1 protein Correlation between SUR1 and neuronal injury.
2) Correlation of the neuroinflammatory cellular responses to the expression of the SUR1 and TRPM4 proteins in the brain regions after direct delivery of the blast wave to the brain via cranial exposure
3) Evaluation of the short term (sensory- and vestibulomotor) outcomes of the glibenclamide efficacy in blast-TBI.
4) Evaluation of the short term long term (cognitive/learning) outcomes of the glibenclamide efficacy in blast-TBI.

REPORTABLE OUTCOMES: Provide a list of reportable outcomes that have resulted from this research

"Rodent Model of Direct Cranial Blast Injury". Kuehn R, Simard PF, Driscoll I, KeledjianK, Ivanova S, Tosun C, Williams A, Bochicchio G, Gerzanich V, Simard JM. *J Neurotrauma* 2011. PM:21639724

"Exposure of the thorax to a sublethal blast wave causes perivascular neuro-inflammation". Simard, JM., Keledjian, K., Tosun, C., Schwartzbauer, G., Ivanova, S., Gerzanich, V. submitted: 2013 *J. Trauma Acute Care Surgery*.

"Blast TBI: Involvement of Direct and Indirect Injury to the Brain" Military Health System Research Symposium 13-16 August 2012, Fort Lauderdale, Florida.

"Selective Vulnerability of the Foramen Magnum Region to Blast TBI." Adam Pampori, Kaspar Keledjian, Cigdem Tosun, Volodymyr Gerzanich, J. Marc Simard. The 17th annual VAMHCS Research Day, May 6, 2013 at the Baltimore VA Medical Center, Baltimore, MD.

"Selective Vulnerability of the Foramen Magnum Region to Blast TBI." Adam Pampori, Kaspar Keledjian, Cigdem Tosun, Volodymyr Gerzanich, J. Marc Simard. National Capital Area TBI Research Symposium, Apr. 29-30 2013 at Natcher Conference Center of the National Institutes of Health, Bethesda, MD. Oral presentation.

"Invivo Diffusion Kurtosis and MR Spectroscopy Changes Following a Novel Direct Cranial Blast TBI." Jiachen Zhuo, Kaspar Keledjian, Adam Pampori, Volodymyr Gerzanich, J. Marc Simard, Rao Gullapali. National Capital Area TBI Research Symposium, Apr. 29-30 2013 at Natcher Conference Center of the National Institutes of Health, Bethesda, MD. Oral presentation.

CONCLUSION: Summarize the results to include the importance and/or implications of the completed research and when necessary, recommend changes on future work to better address the problem. A "so what section" which evaluates the knowledge as a scientific or medical product shall also be included in the conclusion of the report.

1. Detailed analysis of the de novo expression of the SUR1 and TRPM4 proteins shows that the channel formed by these two molecules is significantly upregulated after cranium only Blast TBI.
2. The areas of the brain that show expression of the SUR1/TRPM4 channel exhibited prominent neuroinflammatory responses in agreement with predicted role of the channel in mediating cell injury.
3. Time course of the SUR1/TRPM4 channel expression after Blast-TBI (3 hours to 7 days) indicates that prospective glibenclamide therapy after Blast TBI has to last at least 1 week after the blast.
4. Study of the short term and long term outcomes after Blast TBI with and without treatment suggests that glibenclamide is beneficial in treatment of the vestibulomotor and cognitive deficits after the Blast-TBI.
5. Prophylaxis treatment with glibenclamide prior to the Blast- TBI is more effective in improving long term cognitive deficits.

REFERENCES: List all references pertinent to the report using a standard journal format (i.e. format used in *Science, Military Medicine*, etc.).

1. Kuehn R, Simard PF, Driscoll I, Keledjian K, Ivanova S, Tosun C, Williams A, Bochicchio G, Gerzanich V, Simard JM. Rodent model of direct cranial blast injury. J Neurotrauma. 2011 Oct;28 (10):2155-69. Epub 2011 Aug 8.

2. Simard JM, Woo SK, Bhatta S, Gerzanich V. Drugs acting on SUR1 to treat CNS ischemia and trauma. *Curr Opin Pharmacol* 2008; 8(1):42-9. PM:18032110

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4. Simard JM, Kilbourne M, Tsymbalyuk O, Tosun C, Caridi J, Ivanova S, Keledjian K, Bochicchio G, Gerzanich V. Key role of sulfonylurea receptor 1 in progressive secondary hemorrhage after brain contusion. J Neurotrauma. 2009 Dec;26(12):2257-67. doi: 10.1089/neu.2009.1021.

5. Simard JM, Geng Z, Woo SK, Ivanova S, Tosun C, Melnichenko L, Gerzanich V. Glibenclamide reduces inflammation, vasogenic edema, and caspase-3 activation after subarachnoid hemorrhage. J Cereb Blood Flow Metab. 2009 Feb;29(2):317-30. doi: 10.1038/jcbfm.2008.120. Epub 2008 Oct 15.

SUPPORTING DATA: All figures and/or tables shall include legends and be clearly marked with figure/table numbers